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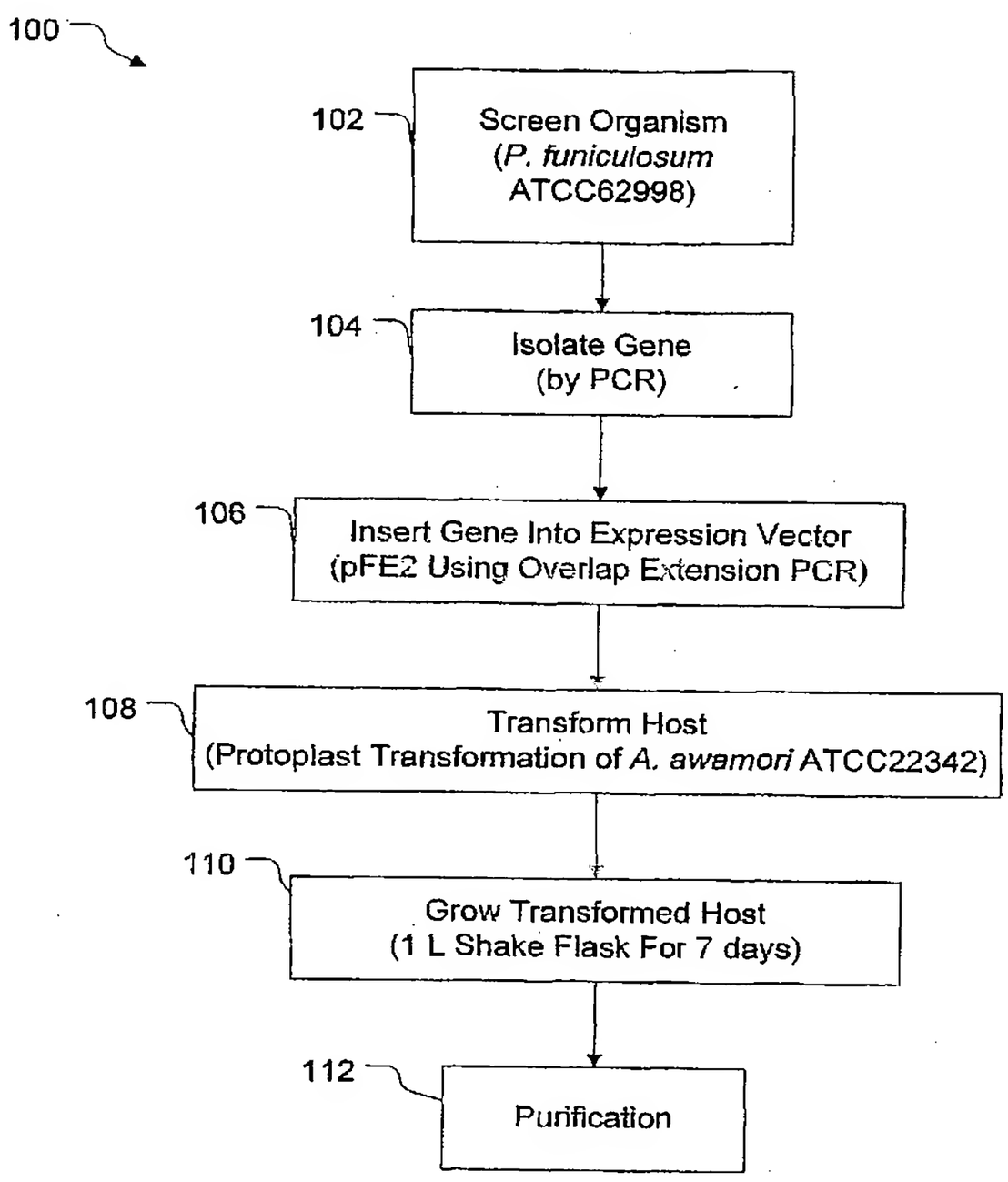
(54) Title: SUPERACTIVE CELLULASE FORMULATION USING CELLOBIOHYDROLASE-1 FROM *PENICILLIUM FUNICULOSUM*



(57) Abstract: Purified cellobiohydrolase I (glycosyl hydrolase family 7 (Cel7A) enzymes from *Penicillium funiculosum* demonstrate a high level of specific performance in comparison to other Cel7 family member enzymes when formulated with purified E1cd endoglucanase from *A. cellulolyticus* and tested on pretreated corn stover. This result is true of the purified native enzyme, as well as recombinantly expressed enzyme, for example, that enzyme expressed in a non-native *Aspergillus* host. In a specific example, the specific performance of the formulation using puriified recombinant Cel7A from *Penicillium funiculosum* expressed in *A. awamori* is increased by more than 200% when compared to a formulation using purified Cel7A from *Trichoderma reesei*.

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SUPERACTIVE CELLULASE FORMULATION USING CELLOBIOHYDROLASE-1 FROM *Penicillium funiculosum*

Contractual Origin of the Invention

The United States Government has rights in this invention under Contract No. DE-AC36-99GO-10337 between the United States Department of Energy and the National Renewable Energy Laboratory, a division of Midwest Research Institute.

Technical Field

The invention pertains to the field of cellulases and, particularly, members of the glycosyl hydrolase Cel7 family, which is also known as the cellobiohydrolase I (CBH I) family of enzymes. More particularly, novel formulations using either purified native *P. funiculosum* Cel7A or purified recombinant *P. funiculosum* Cel7A (rCel7A) expressed from a transgenic *Aspergillus* host exhibit superior performance on pretreated corn stover compared with that formulation using purified *Trichoderma reesei* Cel7A.

Background Art

Cellulosic biomass is a favorable feedstock for fuel ethanol production because it is both readily available and less expensive than either corn or sugarcane. Nevertheless, substantial problems must be overcome before a typical cellulosic feedstock can be utilized effectively and economically as a substrate for the fermentative production of ethanol. By way of example, cellulosic biomass feedstocks may include wood pulp or agricultural residues, such as corn stover, straw, grass, or weeds. A typical feedstock is comprised of approximately 35-45% cellulose, 30-40% hemicellulose, 15% lignin and 10% of other components. The cellulose fraction is composed of linear (and to a substantial extent, microcrystalline) polymers of the hexose sugar, glucose. Saccharification of cellulose releases sugars, which may be converted into ethanol or other products by fermentation. The hemicellulose fraction is comprised mostly of pentose sugars, including xylose and arabinose.

Alcohol products derived from cellulosic biomass are relatively expensive when compared to analogous fuels from other sources. A significant cost factor is the need to provide hydrolyzing enzymes, such as cellulases, that attack the cellulosic and/or

hemicellulosic substrates to release sugars. These enzymes are produced by microorganisms, and may be purified from fermentation broth. The cost of cellulase is presently a significant component of the overall cost of biomass-derived ethanol. In the United States, ethanol production is heavily subsidized by tax incentives that encourage the use of ethanol in reformulated gasoline.

A variety of cellulases are known. Table 1 below lists various cellulases of the Cel7 family. Cel7 enzymes are the principal component in commercial cellulase formulations--typically accounting for most of the actual bond cleavage in the saccharification of cellulose. Cel7 cellobiohydrolases are members of the Class of beta proteins, the Superfamily of concanavalin A-like lectins/glucanases, and the Family of glycosyl hydrolase family 7 catalytic core proteins. The Cel7 family of enzymes may differ from one another by various insertions, deletions, and alterations in the catalytic domain and linker peptide. The cellulose binding domain of Cel7 enzymes is highly conserved. Cel7A from *Trichoderma reesei* is the most widely used CBH I commercial enzyme because it is capable of withstanding commercial process conditions and demonstrates the highest known level of saccharification in the entire family.

TABLE 1

VARIOUS CELLULOSE 1,4-β-CELLOBIOSIDASE MEMBERS

Enzyme	Organism	**GenBank/GenPept Accessions**		**Swiss Prot**
Cellobiohydrolase I	*Agaricus bisporus*	Z50094	CAA90422.1	**Q92400**
exoglucanase C1	*Alternaria alternata*	AF176571	**AAF05699.1**	
Cellobiohydrolase I	*Aspergillus aculeatus*	AB002821	BAA25183.1	**O59843**
Cellobiohydrolase (CbhA)	*Aspergillus nidulans*	AF420019	**AAM54069.1**	
Cellobiohydrolase (CbhB)	*Aspergillus nidulans*	AF420020	**AAM54070.1**	
Cellobiohydrolase A (CbhA)	*Aspergillus niger*	AF156268	**AAF04491.1**	
Cellobiohydrolase B (CbhB)	*Aspergillus niger*	AF156269	**AAF04492.1**	Q9UVS8
Cellobiohydrolase I	*Claviceps purpurea*	Y07550	CAA68840.1	**O00082**

Cellobiohydrolase I	*Cochliobolus carbonum*	U25129	AAC49089.1	**Q00328**
Cellobiohydrolase I	*Cryphonectria parasitica*	L43048	AAB00479.1	**Q00548**
Cellobiohydrolase I (Cel7A)	*Fusarium oxysporum*	L29379	AAA65587.1	**P46238**
Cellobiohydrolase 1.2	*Humicola grisea*	U50594	AAD11942.1 AAN19007.1	**O94093**
Cellobiohydrolase I	*Humicola grisea*	D63515 X17258	BAA09785.1 CAA35159.1	**P15828** Q12621
Cellobiohydrolase I	*Humicola grisea var. thermoidea*	AB003105	BAA74517.1	**O93780**
Cellobiohydrolase I.2	*Humicola grisea var. thermoidea*	AF123441	**AAD31545.1**	
Cellobiohydrolase I	*Melanocarpus albomyces*			
Cellulose 1,4-β-cellobiosidase (Cel7B)	*Melanocarpus albomyces*	AJ515705	**CAD56667.1**	
Cellobiohydrolase I	*Neurospora crassa*	X77778	CAA54815.1	**P38676**
Cellobiohydrolase	*Penicillium funiculosum*	AJ312295	**CAC85737.1**	
Cellobiohydrolase I	*Penicillium janthinellum*	S56178 X59054	CAA41780.1 CAA41780.1	**Q06886**
Cellobiohydrolase	*Phanerochaete chrysosporium*	S40817 X54411	AAA09708.1 CAA38274.1	**Q01762**
Cellobiohydrolase I-1	*Phanerochaete chrysosporium*	M22220 Z22528	AAB46373.1 CAA80253.1	**P13860**
Cellobiohydrolase I-2 (Cel7D)	*Phanerochaete chrysosporium*	L22656 Z11726 Z11733 Z22527 Z29653	AAA19802.1 CAA77789.1 CAA77795.1 CAA80252.1 CAA82761.1 CAA82762.1	**Q09431**
Cellobiohydrolase 1 (Cbh1A)	*Talaromyces emersonii*	AF439935 AY081766	AAL33603.2 **AAL89553.1**	
Cellobiohydrolase I (Cel7A)	*Trichoderma reesei (Hypocrea jecorina)*	X69976	CAA49596.1	**P00725**
Cellobiohydrolase I	*Trichoderma viride*	X53931	CAA37878.1	**P19355**
Cellobiohydrolase I	*Trichoderma viride*	AB021656	BAA36215.1	**O93832**
Cellobiohydrolase I (CbhI)	*Volvariella volvacea V14*	AF156693	**AAD41096.1**	

Cellulases often demonstrate enzymatic synergy in mixtures with other hydrolyzing enzymes; for example, between one enzyme that attacks cellulose and another that attacks hemicellulose. Various efforts have been made to provide transgenic organisms with one or more recombinant genes and obtain multiple functionality from a single organism, for example, as described in United States Patent No. 5,536,655 issued to Thomas et al. for the gene encoding EI endoglucanase from *Acidothermus cellulolyticus*.

United States patent application publication US 2002/0155536 to Van en Brink et al. discloses a method of isolating DNA sequences coding for one or more proteins of interest advantageously using an *Aspergillus* host. More specifically, cDNA is prepared from an organism of interest. Fragments of the cDNA are inserted into a vector to obtain a cDNA library. Subsequent transformation of the cDNA library into filamentous fungi, such as *Aspergillus*, facilitates screening for clones that express proteins of interest.

The '536 patent publication describes an expression system using filamentous fungi, such as *Aspergillus*, to provide host cells to screen for proteins of interest. Expression in an *Aspergillus* host renders the cloned polypeptide sequences more easily detectable due to a higher secretory capacity and less glycosylation, as by way of example, in *Aspergillus niger* as compared to yeast. The '536 patent does not teach that *P. funiculosum* Cel7A can be secreted from *Aspergillus awamori* with full functionality.

United States patent application publication US 2002/0061560 to Lawlis describes a method of obtaining a secretory protein at a higher level in filamentous fungi, for example, *Aspergillus awamori*. More specifically, the coding sequence for the protein of interest is fused with DNA fragments encoding signal peptide, a cleavable linker peptide, and a portion of a protein native to the filamentous fungal host (i.e., protein that is normally secreted from *Aspergillus*). The '560 publication pertains to increased quantities of secreted proteins, and does not teach that *P. funiculosum* Cel7A can be secreted with full functionality.

WO 92/06209 to Ward et al. relates to an improved process for transforming the filamentous fungus *T. reesei*. *T. reesei* cells are treated with homologous DNA originally derived from *T. reesei*. The homologous DNA is provided with a selectable marker, which is used to select transformants. Although CBH I is used as an example, nothing related specifically to the processing and secretion of *P. funiculosum* Cel7A is taught or disclosed.

Efforts in recombinant technologies that pertain to the production of cellulases emphasize the production of cellulase in greater quantity or the production of cellulase having greater activity measured as conversion efficiency over time. It is notoriously difficult to compare the activity or performance of cellulases on naturally occurring cellulosic substrates. The naturally occurring substrates vary in composition, which makes it difficult to provide a uniform basis of comparison. Additionally, one is prone to draw unwarranted conclusions where higher concentrations of enzymes may produce surface effects when the enzyme interferes with itself. Similarly unwarranted conclusions may be drawn where adsorption effects (i.e., enzyme loss) impair the activity of lower cellulase concentrations. When it becomes necessary to measure performance with exactitude, commercial enterprises often choose to consult institutions, such as the National Renewable Energy Laboratory located in Golden, Colorado.

Transgenic expression of genes does not necessarily result in the production of useful cellulase. For example, glycosylation by yeast used to express the Cel7 family enzymes may render the enzymes less effective or ineffective. The choice of host organism is limited to those organisms that can survive commercial process conditions, for example, the Direct Microbial Conversion (DMC) process or the Simultaneous Saccharification and Fermentation (SSF) process. In the DMC method, a single microbial system both produces cellulase and ethanol as a fermentation product. The SSF method utilizes two biological elements, one that produces cellulase enzyme and the other, which ferments sugar to ethanol. The DMC process is described in (Brooks *et. al.*, Proc. Annu. Fuels Biomass Symp., 2nd (1978). The SSF process is described in Ghose *et. al.*, Biotechnol. Bioeng., (1984), 26 (4): 377-381(1984)., e.g., as described by Spindler et al, Biotechnology Letters, 14:403-407 (1992). By way of example, SSF process conditions may impose a pH of 4.5 to 5.5 and a temperature from 30°C to 38°C. It can be difficult to choose a suitable host capable of both expressing a useful form of the cellulase of interest and surviving the process conditions where the cellulase is also active under process conditions.

Summary

The present invention advances the art and overcomes the problems outlined above by providing a cellobiohydrolase I family 7 (Cel7) enzyme formulation using the native

Cel7A from *Penicillium funiculosum* or its recombinant analogue expressed in a transgenic filamentous fungi, e.g., *Aspergillus* sp. The Cel7A enzyme formulation demonstrates an unusually high (if not unprecedented) level of specific performance in saccharification of cellulose.

The Cel7 enzyme formulation contains Cel7A enzyme obtained from *P. funiculosum* or a recombinant analog thereof, for example, a polypeptide having a sequence of SEQ ID NO. 9. The Cel7A enzyme may be combined with an endoglucanase present in amount ranging from one percent (1%) to twenty percent (20%) of the molar concentration of the Cel7A enzyme.

The Cel7A enzyme may be a recombinantly expressed enzyme from a transgenic filamentous fungus. Examples of the transgenic filamentous fungus include *Aspergillus sp.*, *Trichoderma sp.*, and *Penicillium sp.* Further examples of the transgenic filamentous fungus include *Aspergillus awamori, Aspergillus niger, Trichoderma reesei*, and even *Penicillium funiculosum* expressing a transgene encoding the Cel7 enzyme under control of a promoter that is non-native to *Penicillium funiculosum*.

The cellobiohydrolase formulation exhibits specific performance in saccharification of biomass cellulose that exceeds by at least two-fold (i.e., 200%) the specific performance of a comparable cellobiohydrolase formulation where Cel7A cellulase from *Trichoderma reesei* is substituted for the Cel7A enzyme. This level of specific performance is heretofore unknown from Cel7 family enzymes. By way of example, the specific performance may be measured on pretreated corn stover.

The endoglucanase may be EI endoglucanase mixed with the Cel7 enzyme, for example, in a 95:5 molar ratio of the Cel7A enzyme to EI endoglucanase. Additional hydrolyzing enzymes, such as β-glucosidase, may be added to the mixture.

Recombinant expression of the Cel7 may be achieved by transforming a filamentous fungus with a gene from *Penicillium funiculosum*. The gene encodes a Cel7 enzyme that is foreign to the filamentous fungus and/or places the coding region under the control of a non-native promoter. The recombinant Cel7 (rCel7) is expressed by the filamentous fungus using biological processes that operate on the nucleic acid sequence to produce rCel7. The rCel7

enzyme may be purified, e.g., by chromatography, to a concentration that exceeds a concentration obtainable from natural expression processes.

A specific embodiment of the instrumentalities described herein should not be interpreted to define the invention in unduly narrow terms. This is because the specific embodiment teaches by way of example, and not by limitation. *Aspergillus awamori* is used as a host organism to express a particular form of rCel7A enzyme, namely, using a gene encoding Cel7A isolated from *Penicillium* and, more particularly, from *Penicillium funiculosum*.

The Cel7A enzyme represented by SEQ ID NO. 9, after expression *by A. awamori*, is characterized by a thermal denaturation temperature of 66.8°C, as measured by differential scanning microcalorimetry (DSC) at pH 5.0 and a scan rate of 60°C/hour.

Standards for measuring specific performance and other characteristics of cellulases are known in the art. For example, useful techniques of this type are taught by "Hydrolysis of Cellulose Using Ternary Mixtures of Purified Cellulases," J.O. Baker, C.I. Ehrman, W.S. Adney, S.R. Thomas, and M.E. Himmel, *Appl. Biochem. Biotechnol.*, **70/72**, 395-403, (1998); and "A Membrane-Reactor Saccharification Assay to Evaluate the Performance of Cellulases Under Simulated SSF Conditions," J.O. Baker, T.B. Vinzant, C.I. Ehrman, W.S. Adney, and M.E. Himmel, *Appl. Biochem. Biotechnol.*, **63-65**, 585-595, (1997).

Different substrates may be used in testing the Cel7 enzyme formulation. For example, pretreated wood pulp or pretreated agricultural residues may be used for comparative testing purposes to confirm the superior specific performance. Pretreated corn stover is often used as a basis of comparison. Efficient enzymatic hydrolysis of these substrates by cellulase often requires the synergistic cooperation of at least two types of enzymes, namely, endoglucanases and exoglucanases, such as cellobiohydrolase. According to these protocols, the specific performance may be measured using a mixture of enzymes, for example, a mixture of rCel7A (or native Cel7A) and endoglucanase at a molar ratio of 95:5.

Increased specific performance of Cel7A may be facilitated in reaction processes by the addition of other hydrolyzing enzymes, such as β-glucosidase. A particularly useful enzyme mixture includes *T. reesei* Cel7A mixed in a 95:5 molar ratio with *A. cellulolyticus*

EIcd endoglucanase, such as EI endoglucanase expressed from the *Acidothermus cellulolyticus* gene reported in United States Patent No. 5,536,655 issued to Thomas et al., which is incorporated by reference herein. Another useful additional enzyme is β-glucosidase from *Aspergillus niger*, as reported in "Hydrolysis of Cellulose Using Ternary Mixtures of Purified Cellulases," J.O. Baker, C.I. Ehrman, W.S. Adney, S.R. Thomas, and M.E. Himmel, *Appl. Biochem. Biotechnol.*, **70/72**, 395-403, (1998). This formulation shows superior performance. Other hydrolyzing enzymes that may be included in such mixtures include members of Family Cel6.

The *P. funiculosum* rCel7A enzyme may be purified from cultures of native organisms or recombinant hosts. Purification may be accomplished by chromatography to levels of, for example, 50%, 75%, 90%, or research grade purity.

Brief Description of the Drawings

Fig. 1 is a schematic block diagram that shows a process for producing a recombinant Cel7A enzyme;

Fig. 2 shows DNA and polypeptide sequences of interest for Cel7A enzyme isolated from *P. funiculosum*

Fig. 3 depicts a vector that may be recombined with the Cel7 coding region and/or a promoter sequence for use in transforming the filamentous fungus according to the instrumentalities described herein;

Fig. 4 depicts the vector of Fig. 3 after recombination;

Fig. 5 shows a comparison of the specific performance of various Cel7A enzyme formulations (i.e., Cel7A or rCel7A and EIcd endoglucanase) on pretreated corn stover using the DSA assay, where the comparison includes that for standard loadings of purified recombinant Cel7A from *P. funiculosum* expressed in *A. awamori*; purified native Cel7A from *P. funiculosum*; and purified native Cel7A from *T. reesei*. Also illustrated in Fig. 5 is the one-half loading of purified recombinant Cel7A from *P. funiculosum* expressed in *A. awamori*.

Fig. 6 provides a comparison of thermal transition temperatures between various purified Cel7A enzymes using DSC.

Detailed Description

There will now be shown and described a process for the production of rCel7 in *Aspergillus* and, specifically *P. funiculosum* rCel7A expressed in *A. awamori*. The presentation of a specific embodiment according to the various embodiments and instrumentalities described herein should not unduly limit the scope of the invention, because the teaching is by way of example.

Procedures for constructing recombinant molecules are disclosed by Sambrook et al., supra. Briefly, a DNA sequence encoding a Cel7A enzyme from *Penicillium funiculosum* , or its functional derivatives exhibiting Cel7 activity, may be recombined with vector DNA in accordance with conventional techniques. These techniques and materials include, for example, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and blunt-ended or cohesive-ended ligation with appropriate ligases. Part or all of the genes may be synthesized chemically in overlapping fragments which are hybridized in groups and ligated to form longer double-stranded DNA molecules. The resulting vector may then be introduced into a host cell by transformation, transfection, techniques such as electroporation, etc. Techniques for introducing a vector into a host cell are well known.

A vector is a DNA molecule, often derived from a plasmid, bacteriophage or hybrid, into which fragments of DNA may be inserted or cloned. A vector usually contains one or more unique restriction sites, and may be capable of autonomous replication or integration into the genome of a defined host organism such that the cloned sequence is reproducible.

Fig. 1 is a process diagram 100 showing steps for the production of rCel7A from *P. funiculosum*. In step 102, a culture of a source organism, such as the source organisms identified above in Table 1, are screened for Cel7 activity. For example, *P. funiculosum* (ATCC62998) is screened to identify the presence of Cel7A. In step 104, primers are constructed based upon the presumed commonality of conserved sequences among the respective domains, such as the catalytic domain in the Cel7A family. The gene is then isolated by polymerase chain reaction (PCR). Step 106 entails inserting the gene into an expression vector, for example, using overlap extension PCR to insert the gene into expression vector pFE2. In step 108, the expression vector is used in host transformation,

specifically protoplast transformation of *Aspergillus awamori* ATCC22342. A transformed strain that is confirmed to carry the rCel7A gene is grown for a period of time in step 110, and the rCel7A is purified in step 112.

The Cel7A family genes can be cloned for expression in *Aspergillus* and other filamentous fungi using recombinant DNA techniques, as will be described below and illustrated by specific example. Variations on laboratory techniques are well known and may be adapted to implement the instrumentalities described herein. In addition to the disclosed embodiments, the DNA cloning process can be facilitated through a variety of other means, such as application of recombinant DNA techniques, the polymerase chain reaction techniques (PCR) or DNA synthesis of the gene. Techniques for synthesizing oligonucleotides are disclosed by, for example, Wu et al, Prog. Nucl. Acid. Res. Molec. Biol. 21:101-141 (1978).

Standard reference works setting forth the general principles of recombinant DNA technology and cell biology include, for example, Watson et al., Molecular Biology of the Gene, Volumes I and II, Benjamin/Cummings Publishing Co., Inc., Menlo Park, Calif. (1987); Darnell et al., Molecular Cell Biology, Scientific American Books, Inc., New York, N.Y. (1986); Lewin, Genes II, John Wiley & Sons, New York, N.Y. (1985); Old et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2nd Ed., University of California Press, Berkeley, Calif. (1981); Sambrook et al, (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989)) and Albers et al., Molecular Biology of the Cell, 2nd Ed., Garland Publishing, Inc., New York, N.Y. (1989).

The following nonlimiting examples establish preferred materials and methods for practicing the process steps of Fig. 1, as well as for use in comparing the specific activities of rCel7A enzymes

EXAMPLE 1

ISOLATING A Cel7A GENE FROM *P. funiculosum*

The coding sequence for the *cel7A* gene from *P. funiculosum* (ATCC 62998) (SEQ ID NO. 7 of Fig. 2) was inserted and expressed in the fungal host *Aspergillus awamori* (ATCC 22342) using the fungal expression vector pFE2. As shown in Fig. 3, the pFE2

vector is an *E. coli* - *Aspergillus* shuttle vector, and contains elements required for maintenance in both hosts. The pFE2 vector directs the expression and secretion of the protein of interest as a fusion protein with a portion of the glucoamylase (GA) gene fused to the *cel7A* gene. The vector contains the *Streptoalloteichus hindustanus* phleomycin resistance gene (Phleo) under the control of the *A. niger* β-tubulin promoter, for positive selection of *Aspergillus* transformants. The vector also contains a β-lactamase gene for positive selection using ampicillin in *E. coli*, and the *A. niger* trpC terminator (trpC T). In this case, the rCel7 protein from *P. funiculosum* was expressed with its own secretion signal peptide by replacing the glucoamylase signal by PCR overlap extension and was expressed under the control of the *Aspergillus niger* GA promoter.

As shown in Fig. 4, the construct used to produce recombinant Cel7 enzyme was designated pFE*cbhI*(Pf)3726 and was designed to contain the native *P. funiculosum* signal sequence (SEQ ID. NO. 8; Pf signal) with the native coding sequence (*P. funiculosum cbhI* gene) for the structural protein. The 1.5-kb *P. funiculosum cbhI* structural gene (including the signal sequence starting with ATG codon and the mature protein coding sequence, which ends with TAG stop codon) was used to replace the GA signal sequence in the pFE2 vector. The GA signal sequence was replaced using overlap extension PCR where the native *P. funiculosum* structural gene was placed precisely under the GA promoter and regulatory region in pFE2. In addition, a NotI restriction site was added after the TAG codon (see primer Pf*cbhI*NotI(R)) to position the gene immediately before the trpC T terminator region in pFE2.

The regulatory region containing GA promoter and native *P. funiculosum* Cel7A signal region (0.9 kb) was PCR amplified using PfuI with the following oligonucleotide primers:

FE2-2(F): 5'GTATACACGCTGGATCCGAACTCC 3' (SEQ ID NO. 1)

FE2-Pf*cbhI*(R):

5'GTTCAAGGCAGACATTGCTGAGGTGTAATGATGC 3' (SEQ ID NO. 2)

[5'Pf*cbhI* signal(tail)] [Sequence before GA signal]

The template used for the PCR was pFE2 vector. The bolded region of SEQ ID NO. 2 above anneals to the sequence immediately before the GA signal sequence in pFE2. The underlined region is complementary to the 5' *P. funiculosum* CBH I signal coding sequence. After this round of PCR, the regulatory region included the GA promoter region from pFE2 and 5'end of native *P. funiculosum* Cel7A signal sequence.

The structural gene of *P. funiculosum* Cel7A was amplified using the following primers:

Pf*cbhI*-FE2(F):

5'**GCATCATTACACCTCAGCA**ATGTCTGCCTTGAAC 3' (SEQ ID NO. 3)

[Sequence before GA signal (tail)] [5'Pf CBH I signal]

Pf*cbhI*NotI(R):

5'ATAAGAATGCGGCCGCCTACAAACATTGAGAGTAGTAAGGG 3' (SEQ ID NO. 4).

Genomic DNA of *P. funiculosum* (ATCC 62998) was used as template for the PCR reaction. The bolded sequence in Pf*cbhI*-FE2 (F) primer (SEQ ID NO. 3) annealed with pFE2 vector and not the *P. funiculosum cel7A* coding sequence. Following this round of PCR the 5'-end of the *cel7A* structural gene contained the extra sequence of the GA signal.

To combine the GA promoter and GA regulatory region from pFE2 and the *P. funiculosum cel7* structural gene, a third round of PCR was conducted using the two PCR products described above and primers FE2-2 (F) (SEQ ID NO. 1) and Pf*cbhI*NotI(R) (SEQ ID NO. 4). The product (2.6 kb) was ligated in a T/A cloning vector, pGEMTeasy, which is obtainable on commercial order from Promega of Madison, Wisconsin, and used to transform *E. coli* DH5α. Transformants with correct inserts were identified by colony PCR screening using a primer pair including:

Pf3 (F): ACTTCGTTACCGGCTCTAACG (SEQ ID NO. 5) and

Pf4(R): GAAGTCACATCCGTCAGGGTC (SEQ ID NO. 6).

The primer pair Pf3 (F) (SEQ ID NO. 5) and Pf4(R) (SEQ ID NO. 6) is internal to *P. funiculosum* Cel7A. Plasmid DNA was extracted from several of these correct transformants and digested with BamHI followed by Klenow polymerase treatment to generate the blunt ends. BamHI is located in the 5'end of the 2.6-kb fragment and intrinsic to the GA promoter region of pFE2 vector. A 2.4-kb fragment was obtained by NotI digestion of the BamHI/Klenow linearized plasmid. NotI was included in the Pf*cbhI*NotI(R) for cloning purposes. To transfer this 2.4-kb fragment into pFE2, pFE2 was digested with SacI and blunt-ended with T4 DNA polymerase followed by digestion with NotI. A 5.5-kb vector fragment was recovered by gel extraction and ligated to the 2.4-kb fragment. The recombinant plasmid was named pF*cbhI*(Pf)3726.

After transformation in DH5α and confirmation of the plasmid by colony PCR and restriction digestion pattern, the plasmid was used to transform *A. awamori* by spheroplast transformation. Zeocin-resistant transformants were grown in liquid medium CMZ300 (CM+Zeocin 300ug/mL) for 3-4 days and supernatant analyzed by activity on p-nitrophenol β-D lactopyranoside activity assay. One of the positive clones was grown in 1-L CMZ300 medium for 7-10 days and supernatant subjected to protein purification and analysis. Expression products of this clone were confirmed to contain the recombinant Cel7A enzyme isolated from *P. funiculosum* (SEQ ID NO. 9).

EXAMPLE 2

TRANSFORMING *A. awamori*

Aspergillus awamori (ATCC22342) spore stocks were stored at –70°C in 20% glycerol, 10% lactose. After thawing, 200 µL of spores were inoculated into 50 mL CM-glucose broth (5 $g \cdot L^{-1}$ Yeast Extract; 5 $g \cdot L^{-1}$ Tryptone; 10 $g \cdot L^{-1}$ Glucose; 20X Clutterbuck's Salts (120.0 $g \cdot L^{-1}$ Na_2NO_3; 10.4 $g \cdot L^{-1}$ KCl; 10.4 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$; 30.4 $g \cdot L^{-1}$ KH_2PO_4) 50 $mL \cdot L^{-1}$) at pH 7.5 in each of eight-baffled 250 mL Erlenmeyer flask. The cultures were grown at 28°C, 225 rpm for 48 h.

The mycelial balls were removed by filtration through sterile Miracloth (Calbiochem, San Diego, CA) and washed *thoroughly* with sterile KCM (0.7M KCl, 10mM MOPS, pH

5.8) to remove ungerminated spores. Approximately 10 g wet weight of washed mycelia were transferred to 50 mL KCM + 250 mg Lysing Enzyme from *Trichoderma harzianum* (Sigma-Aldrich, St. Louis, MO) in a 250 mL baffled Erlenmeyer flask. The digestion mixture was incubated overnight at 30°C, 80 rpm.

Following digestion, the mycelia were titurated with a 25 mL disposable pipette to loosen the hyphal cells and filtered through sterile Miracloth into 50 mL conical centrifuge tubes. The spheroplasts were pelleted at 1500 x g for 12 min and resuspended in 0.7M KCl by gentle tituration with a 25 mL pipette. This was repeated once. After a third pelleting, the spheroplasts were resuspended in 10 mL KC (0.7M KCl; 50mM $CaCl_2$), pelleted and resuspended in 1.0 mL KC using a wide-bore pipet tip.

The washed spheroplasts were transformed by adding 12.5 µL PCM (40% PEG 8000; 50mM $CaCl_2$; 10mM MOPS pH 5.8) and 5 µL DNA (~0.5 µg/µL) to 50 µL of spheroplasts in sterile 1.5 mL Eppendorf tubes. After incubation on ice for 45 minutes, 0.5 mL of room temperature PCM was added to the transformation mixture and was mixed by inversion and gentle vortexing. The mixture was incubated at room temperature for 45 minutes. One milliliter of KC was added and mixed. Selection of transformants was by zeocin resistance. The mixture was allocated between four tubes (10 mL each) of CM top agar at 55°C, which were each poured over a 15 mL CM plate with 170 µg/mL zeocin. The plates were incubated at 28°C for 2-3 days. Subsurface colonies were partially picked with a sterile wide bore pipet tip, exposing the remaining part of the colony to air and promoting rapid sporulation. After sporulation, spores were streaked onto several successive CM plates with either 100 or 300 µg/mL zeocin added. After a monoculture was established, heavily sporulated plates were flooded with sterile spore suspension medium (20% glycerol, 10% lactose), the spores were suspended and aliquots were frozen at –70°C. Working spore stocks were stored on CM slants in screw cap tubes at 4°C. The recombination event was through random integration of the plasmid into the *A. awamori* genome.

EXAMPLE 3

CULTURING *A. awamori*

For enzyme production, spores were inoculated into 50 mL CM basal fermentation medium (5.0 $g \cdot L^{-1}$ Enzymatic Casein Hydrolysate; 5.0 $g \cdot L^{-1}$ NH_4CL; 10.0 $g \cdot L^{-1}$ Yeast Extract; 10.0 $g \cdot L^{-1}$ Tryptone; 2.0 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_20$; 50.0 $g \cdot L^{-1}$ Soluble Starch; 50 mM Bis-Tris-Propane, pH 7.0), and grown at 32°C, 225 rpm in 250 mL baffled flasks. The cultures were transferred to 1.0 L of basal fermentation medium in 2800 mL Fernbach flasks and grown under similar conditions. The flasks were harvested by filtration through Miracloth after 7-10 days of growth.

EXAMPLE 4

PURIFYING rCel7A FROM *A. awamori* CULTURE BROTH

The purification of rCBH I from *A. awamori* fungal broths was started by filtration through glass fiber filters followed by concentration of the broth by using a 500 mL Amicon stirred cell concentrator with PM-10 cutoff filters. After the broth was concentrated to a volume of approximately 50 mL and was then extensively diafiltered by successive dilution and concentration with the stirred cell with 50 mM Bis-Tris pH 5.8 buffer to a point at which the conductivity of the solution was less than 2 mS/cm. The sample was then applied to a HiPrep 16/10 DEAE FF column (Amersham Biosciences) equilibrated with 50 mM Bis-Tris, pH 5.8 buffer with a flow rate of 10 mL/min at 4°C. After the sample was loaded and the column washed extensively with equilibration buffer the bound fraction was eluted with a linear gradient of 0 to 1.0 M NaCl in the same equilibration buffer. The fractions containing activity on p-nitrophenol β-D-lactopyranoside were pooled and concentrated to a final volume of 10 mL using Amicon stirred cell concentrators and PM-10 cutoff filters (10,000 kDa nominal molecular weight cutoff). The enzyme was further purified and the buffer exchanged by means of size exclusion chromatography using a HiLoad 26/60 Superdex 200 column (Amersham Biosciences) in 20 mM acetate, 100 mM NaCl, pH 5.0 buffer. At this point the protein eluted as a single, symmetrical peak and the purity was confirmed as a single band when analyzed with a NuPage 4-12 % Bis-Tris gradient gel using MOPS-SDS buffer (Invitrogen) according to the manufactures recommended conditions. Concentrations of purified proteins were determined by absorbance at 280 nm using the extinction

coefficient and molecular weight calculated for Cel7A by the ProtParam Software obtained from the ExPASy website at http://www.expasy.ch/tools/protparam.html.

EXAMPLE 5

PRODUCTION AND PURIFICATION OF NATIVE *Penicillium funiculosum* Cel7A

Native Cel7A protein was produced from *Penicillium funiculosum* grown in two 500 mL cultures in CM-PSC broth (5 $g \cdot L^{-1}$ Yeast Extract; 10 $g \cdot L^{-1}$ phosphoric acid swollen cellulose; 5 $g \cdot L^{-1}$ Tryptone; 20X Clutterbuck's Salts (120.0 $g \cdot L^{-1}$ Na_2NO_3; 10.4 $g \cdot L^{-1}$ KCl; 10.4 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$; 30.4 $g \cdot L^{-1}$ KH_2PO_4) 50 $mL \cdot L^{-1}$) at pH 7.5 in baffled 1 L Erlenmeyer flasks. The flasks were inoculated with 1 mL of a spore suspension of *P. funiculosum* ATCC 62998 and incubated at 28°C in a New Brunswick Scientific Model Innova 4230 shaking incubator at 225 rpm. The cultures were harvested by filtration following 7 days of growth.

The purification of the native Cel7 from *P. funiculosum* broth was started by filtration through glass fiber filters followed by concentration of the broth by using a 500 mL Amicon stirred cell concentrator with PM-10 cutoff membranes. The broth was first concentrated to a volume of approximately 50 mL followed by extensive diafiltration by successively diluting and concentrating the broth with 50 mM Bis-Tris pH 5.8 buffer to a point at which the conductivity of the solution was less than 2 mS/cm. The sample was then applied to a 6 mL Resource Q column (Amersham Biosciences) equilibrated with 50 mM Bis-Tris, pH 5.8 buffer with a flow rate of 6 mL/min. After the sample was loaded and the column washed extensively with equilibration buffer and the bound fraction was eluted with a linear gradient of 0 to 1.0 M NaCl in the same equilibration buffer. The fractions containing activity on p-nitrophenol β-D lactopyranoside were pooled and concentrated to a final volume of 10 mL using Amicon stirred cell concentrators and PM-10 cutoff filters (10,000 kDa nominal molecular weight cutoff). The enzyme was further purified and the buffer exchanged by means of size exclusion chromatography using a HiLoad 26/60 Superdex 200 column (Amersham Biosciences) in 20 mM acetate, 100 mM NaCl, pH 5.0 buffer. The purity was confirmed as a single band using a NuPage 4-12 % Bis-Tris gradient gel and MOPS-SDS buffer (Invitrogen) according to the manufacturer's recommended conditions. The concentration of the purified native Cel7 solution was determined by absorbance at 280 nm

using the extinction coefficient and molecular weight calculated for Cel7A by the ProtParam tool on the ExPASy website (http://www.expasy.ch/tools/protparam.html).

EXAMPLE 6

COMPARATIVE TESTING OF SPECIFIC PERFORMANCE

The purified rCel7A and Cel7A from Examples 4 and 5 were tested against other Cel7A enzymes. Test procedures included those described in "Hydrolysis of Cellulose Using Ternary Mixtures of Purified Cellulases," J.O. Baker, C.I. Ehrman, W.S. Adney, S.R. Thomas, and M.E. Himmel, *Appl. Biochem. Biotechnol.*, **70/72**, 395-403, (1998), and "A Membrane-Reactor Saccharification Assay to Evaluate the Performance of Cellulases Under Simulated SSF Conditions," J.O. Baker, T.B. Vinzant, C.I. Ehrman, W.S. Adney, and M.E. Himmel, *Appl. Biochem. Biotechnol.*, **63-65**, 585-595, (1997).

Diafiltration saccharification assays (DSA) were performed as disclosed in the above article by Baker et al. (1997) with the modifications that the membrane installed in the cells was a BioMax-5 (5,000 Da nom. MWCO, Millipore Corp.) rather than a PM-10 (10,000 Da nom. MWCO, Amicon, Inc.), and the buffer flow rate through the membrane was 0.020 mL/min. All assays were at pH 5.0 in acetate buffer with 0.02%(w/v) sodium azide added. Assays were run at 38°C as a compromise between the higher activities of the cellulases at still higher temperatures and the temperature-tolerance of *S. cerevisiae* D5A, the organism used in companion simultaneous saccharification and fermentation (SSF) assays.

Pretreated corn stover (1% wt/wt sulfuric acid for 2-4 min at 190°C) was prepared for use as a DSA substrate. Substrate loadings averaged 96.4 mg (dry wt.) biomass (standard deviation, n = 5, of 0.8% or less) per DSA cell loading, for cellulose loadings of 55.5 mg glucose per assay. Substrate loadings thus amounted to 4.3% (w/v, solids) or 2.5% (w/v, cellulose). Because the effective saccharification of crystalline cellulose requires the synergistic action of both endoglucanases and exoglucanases, the activities of Cel7A and rCel7A proteins were assayed in combination with the catalytic domain of *A. cellulolyticus* endoglucanase I (EIcd), used here as a standard endoglucanase. In all DSA protocols, the particular Cel7A species being tested was loaded at 27.8 mg per g cellulose, and the endoglucanase (EIcd) was loaded at 1.11 mg per g cellulose, resulting in a 95:5 molar ratio of

exoglucanase to endoglucanase. Quantitation of product sugars in effluent fractions was by HPLC using an Aminex HPX-87H column operated at 65°C with 0.01 N H_2SO_4 (0.6 mL/min) as mobile phase, in an Agilent Model 1100 chromatograph.

Protein Stability Measurements

The overall protein stability of rCel7A from Example 4 was measured by differential scanning microcalorimetry using a Microcal model VP-DSC calorimeter (Microcal, Inc., Northampton, MA), with data analysis by means of Origin for DSC software (Microcal). Thermograms were collected for samples containing 50 µg/mL protein at pH 5.0 in 20 mM sodium acetate with 100 mM NaCl. Calorimeter scan rate was 60°C/h.

Fig. 5 shows comparison saccharification results as a plot of percent conversion of pretreated corn stover over time. Saccharification conditions included a pH of 5.0, a temperature of 38°C, and a standard enzyme loading of Cel7A at 27.8 mg/g cellulose plus E1 endoglucanase at 1.13 mg/g cellulose. The comparison was between the specific performance of (A) native Cel7A from *T. reesei*, (B) native Cel7A from *P. funiculosum*, (C) rCel7A from *P. funiculosum* expressed in *A. awamori*, and (D) a one-half loading of rCel7A from *P. funiculosum* expressed in *A. awamori*.

The results shown in Fig. 5 demonstrate advantages that are heretofore unknown in the art. Native *T. reesei* Cel7A is the current industry standard Cel7A enzyme used in cellulase formulations for biomass conversion. Surprisingly, an equivalent loading (27.8 mg protein/g cellulose) of purified Cel7A from *P. funiculosum* converted the pretreated corn stover substrate much more readily, with an approximate 69% conversion of cellulose being obtained at 72 hours, versus 52% conversion for the native *T. reesei* Cel7A. Furthermore, an equivalent loading of rCel7A from *P. funiculosum* expressed in *A. awamori* also yielded unexpectedly high performance, with 65% conversion of cellulose in 72 hours. This is important for process scale production of Cel7A, because heterologous expression of the *P. funiculosum* Cel7A from filamentous hosts is a viable large-scale production strategy. Fig. 5 also shows that at one-half loading (i.e., 13.9 mg protein/g cellulose) of the rCel7A *P. funiculosum* enzyme expressed in *A. awamori*, the performance on pretreated corn stover is still much greater than that of the native *T. reesei* Cel7A enzyme. These results show that the new *P. funiculosm* Cel7A + *A. cellulolyticus* EIcd endoglucanase formulation deliver greater

than 2–fold increase in specific performance relative to the *T. reesei* enzyme. Importantly, the native and recombinant Cel7A from *P. funiculosum* display this specific performance under conditions suitable for commercial SSF processes (pH 5 and 38°C).

In conclusion, the data presented in Fig. 5 confirm that the cellulose digestion kinetics are such that native *P. funiculosum* Cel7A and *P. funiculosum* rCel7A expressed in *A. awamori* outperform native *T. reesei* Cel 7A by a factor of at least two, as demonstrated by the superior performance of *P. funiculosum* rCel7A (13.9 mg protein/g cellulose) relative to *T. reesei* Cel7A (27.8 mg/g cellulose) at all points measured over the interval from 0 to 136 hours.

EXAMPLE 7

CHARACTERIZATION OF *P. funiculosum* rCel7A

The purified rCel7A from Example 6 was tested against other Cel7A enzymes that were not expressed in *A. awamori* to determine the maximal thermal transition temperature. Test procedures included the use of differential scanning calorimetry (DSC) instrumentation.

Fig. 6 shows the comparative DSC results. The rCel7A from *P. funiculosum* expressed in *A. awamori* shows an optimal activation temperature of 66.8°C, as compared to 65.0°C and 68.2°C, respectively, for native *T. reesei* Cel7A and rCel7A from *T. reesei* expressed in *A. awamori*.

The foregoing discussion is intended to illustrate concepts by way of example with emphasis upon the preferred embodiments and instrumentalities. Accordingly, the disclosed embodiments and instrumentalities are not exhaustive of all options or mannerisms for practicing the disclosed principles hereof. The inventors hereby state their intention to rely upon the Doctrine of Equivalents in protecting the full scope and spirit of the invention.

Claims

1. A cellulase formulation comprising:

a cellobiohydrolase I (glycosyl hydrolase family 7 (Cel7)) enzyme having a polypeptide sequence of SEQ ID NO. 9; and

an endoglucanase present in amount ranging from one percent (1%) to twenty percent (20%) of the molar concentration of the Cel7 enzyme.

2. The cellulase formulation of claim 1, wherein the Cel7 enzyme is a heterologously expressed enzyme from a transgenic filamentous fungus.

3. The cellulase formulation of claim 2, wherein the transgenic filamentous fungus includes *Aspergillus*.

4. The cellulase formulation of claim 3, wherein the *Aspergillus* includes *Aspergillus awamori*.

5. The cellulase formulation of claim 2, wherein the transgenic filamentous fungus includes *Trichoderma*.

6. The cellulase formulation of claim 5, wherein the transgenic filamentous fungus includes *Trichoderma reesei*.

7. The cellulase formulation of claim 2, wherein the transgenic filamentous fungus includes *Penicillium* and a transgene coding for expression of the Cel7 enzyme is under control of a non-native promoter.

8. The cellulase formulation of claim 7, wherein the *Penicillium* comprises *Penicillium funiculosum*.

9. The cellulase formulation of claim 1, wherein the cellobiohydrolase formulation exhibits specific performance in saccharification of cellulose that exceeds by at least two-fold the specific performance of a comparable cellobiohydrolase formulation where Cel7A cellulase from *Trichoderma reesei* is substituted for the Cel7 enzyme.

10. The cellulase formulation of claim 9, wherein the specific performance is measured on corn stover.

11. The cellulase formulation of claim 1, wherein the endoglucanase comprises EIcd endoglucanase .

12. The cellulase formulation of claim 11, wherein the mixture comprises a 95:5 molar ratio of the Cel7A enzyme to EIcd endoglucanase.

13. The cellulase formulation of claim 1, further comprising at least one additional hydrolyzing enzyme.

14. The cellulase formulation of claim 1, wherein the Cel7 enzyme is characterized by a peak activation temperature of 68°C.

15. In a process for producing cellobiohydrolase 1 family 7 (Cel7) enzyme from a recombinant host, the improvement comprising:

transforming a filamentous fungus with a gene from *Penicillium funiculosum*, the gene encoding for a Cel7 enzyme foreign to the filamentous fungus; and

expressing the rCel7A enzyme from the filamentous fungus.

16. The process of claim 15, further comprising a step of purifying the Cel7 enzyme to a concentration than is obtainable from natural expression processes.

17. The process of claim 15 further comprising a step of saccharifying cellulose with the Cel7 enzyme.

18. A purified Cel7 enzyme produced by the process of claim 17.

19. A Cel7A enzyme produced by the process of claim 15.

20. A method of producing a recombinant cellobiohydrolase 1 (rCel7) enzyme, the method comprising the steps of:

obtaining a nucleic acid sequence that encodes a native Cel7A enzyme from *Penicillium funiculosum*.

transforming a filamentous fungus with the nucleic acid sequence; and

expressing rCel7A in the filamentous fungus by biological processes that operate on the nucleic acid sequence to produce rCel7 in the filamentous fungus.

21. The method of claim 20, wherein the filamentous fungus used in the transforming and expressing steps comprises *Aspergillus*.

22. The method of claim 21, wherein the *Aspergillus* comprises *Aspergillus awamori*.

23. The method of claim 20, wherein the filamentous fungus used in the transforming and expressing steps comprises *Trichoderma*.

24. The method of claim 23, wherein the *Trichoderma* comprises *Trichoderma reesei*.

25. The method of claim 24, further comprising a step of mixing the rCel7 enzyme with an endoglucanase.

26. The method of claim 25, wherein the endoglucanase comprises EIcd endoglucanase.

27. The method of claim 26, wherein the step of mixing comprises mixing a 95:5 molar ratio of the rCel7A to EIcd endoglucanase.

28. A rCel7A produced by the method of claim 20.

29. A purified Cel7A cellulase according to SEQ ID NO. 9.

30. The purified Cel7A cellulase of claim 29 at least 50% pure.

31. The purified Cel7A cellulase of claim 29 at least 75% pure.

32. The purified Cel7A cellulase of claim 29 at least 90% pure.

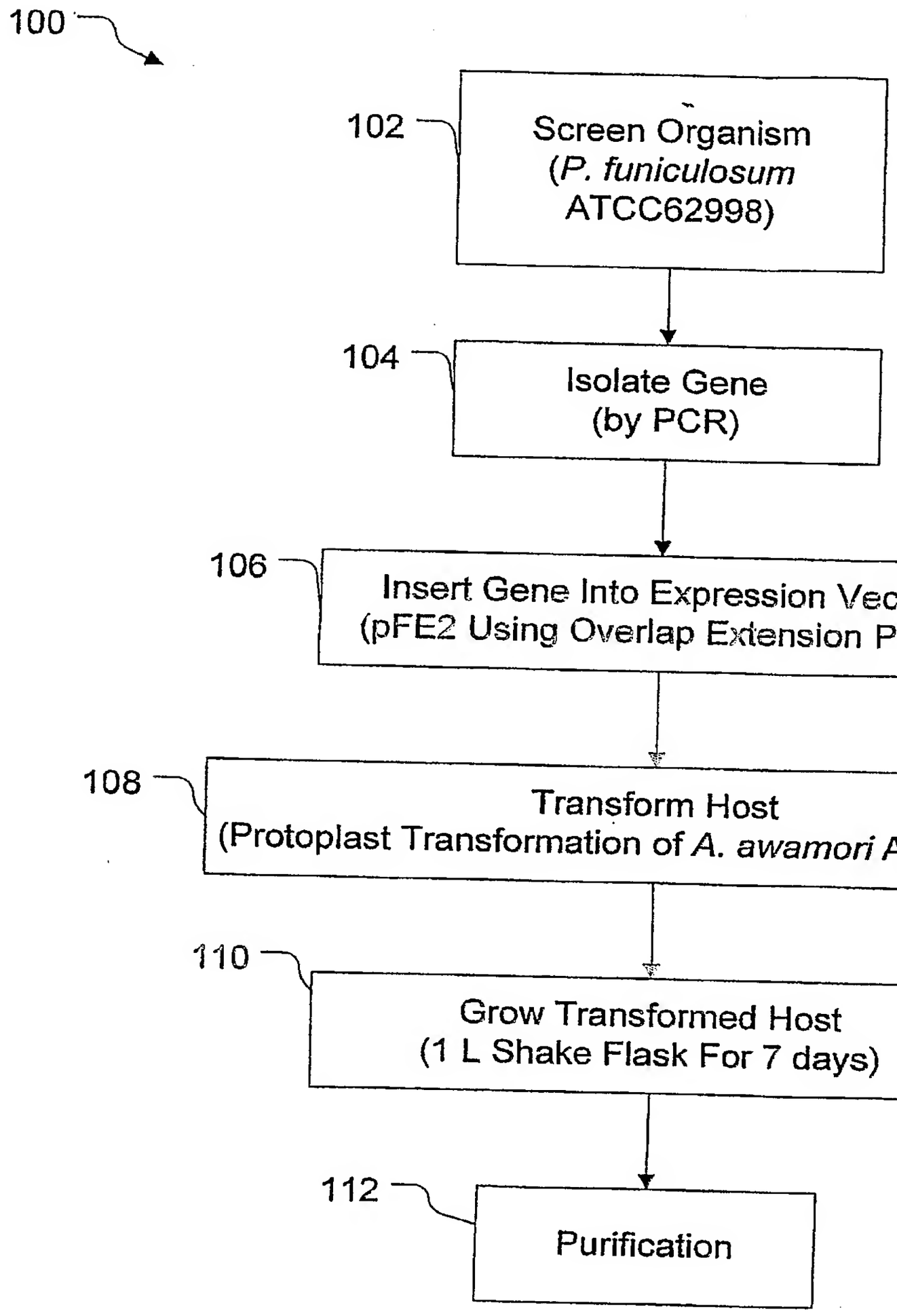

Fig. 1

SEQ. ID NO. 7	Nucleic acid sequence of *P. funiculosum* native cellobiohydrolase I	CAGCAAATTGGTACTTATACCGCCGAAACCCATCCCTCTTTGAGCTGGTCTACTTGTAA ATCGGGTGGTAGTTGCACCACGAACTCCGGTGCCATTACGTTGGATGCCAACTGGCGTT GGGTCCATGGTGTCAATACCAGCACTAACTGCTACACTGGCAACACTTGGAATACCGC CATCTGCGACACTGATGCTTCTTGTGCCCAGGACTGTGCTCTCGACGGTGCTGACTACT CTGGCACATACGGTATCACTACCTCTGGCAACTCGTTGCGCCTGAACTTCGTTACCGGC TCTAACGTTGGATCTCGTACCTACCTGATGGCCGATAACACCCACTACCAGATCTTCGA CTTATTGAACCAAGAGTTCACCTTCACCGTCGATGTCTCTAACCTCCCTTGCGGTTTGA ACGGTGCCCTCTACTTCGTGACCATGGACGCCGACGGTGGTGTCTCCAAGTACCCCAAC AACAAGGCTGGTGCTCAGTACGGTGTTGGATACTGTGACTCTCAATGCCCTCGTGACTT GAAGTTCATCGCTGGTCAGGCCAACGTCGAGGGCTGGACGCCTTCCACCAACAACTCG AACACTGGAATCGGCAACCACGGATCTTGCTGCGCGGAGCTTGATATCTGGGAAGCAA ACAGTATCTCAGAGGCCTTGACTCCTCACCCTTGCGATACACCCGGCCTAACTGTTTGC ACTGCTGATGACTGTGGTGGTACCTACAGCTCCAATCGTTATGCTGGTACCTGCGACCC TGACGGATGTGACTTCAATCCTTACCGTCTCGGTGTCACTGACTTCTACGGCTCCGGCA AGACCGTCGACACCACCAAGCCCTTCACCGTTGTGACTCAATTCGTCACTGACGACGGT ACCTCCAGCGGTTCCCTTTCTGAGATCAGACGTTACTACGTCCAGAACGGCGTTGTCAT CCCCCAGCCTTCCTCCAAGATCTCCGGAATCAGCGGTAATGTTATCAACTCCGACTTCT GCGCTGCTGAGCTCTCCGCCTTTGGCGAGACTGCCTCGTTCACCAACCACGGTGGCTTG AAAAACATGGGCTCTGCTTTGGAAGCTGGTATGGTCTTGGTCATGAGCTTGTGGGACG ACTACTCCGTCAACATGCTCTGGCTCGACAGCACATACCCAGCAAACGAGACTGGTAC CCCCGGTGCTGCTCGTGGTTCCTGCCCTACCACCTCTGGTAACCCCAAGACCGTTGAAT CCCAATCTGGCAGCTCCTATGTGGTCTTCTCTGACATCAAGGTTGGTCCTTTCAACTCTA CTTTCAGCGGTGGTACTAGCACCGGTGGCAGCACTACTACTACTGCCAGTGGCACCACC TCCACTAAGGCCTCCACTACCTCTACTTCCAGCACTTCTACCGGCACTGGAGTCGCTGC TCACTGGGGTCAGTGTGGTGGCCAGGGCTGGACTGGTCCTACCACCTGTGCTAGTGGA ACCACTTGCACCGTTGTGAACCCTTACTACTCTCAATGTTTGTAG
SEQ. ID NO: 8	Nucleic acid sequence *of P. funiculosum* native signal sequence	ATGTCTGCCTTGAACTCTTTCAATATGTACAAGAGCGCCCTCATCTTGGGTTCCTTGCTG GCAACAGCTGGTGCT
SEQ. ID NO: 9	Amino acid sequence of expressed the native *P. funiculosum* cellobiohydrolase	Gln Gln Ile Gly Thr Tyr Thr Ala Glu Thr His Pro Ser Leu Ser 15 Trp Ser Thr Cys Lys Ser Gly Gly Ser Cys Thr Thr Asn Ser Gly 30 Ala Ile Thr Leu Asp Ala Asn Trp Arg Trp Val His Gly Val Asn 45 Thr Ser Thr Asn Cys Tyr Thr Gly Asn Thr Trp Asn Thr Ala Ile 60 Cys Asp Thr Asp Ala Ser Cys Ala Gln Asp Cys Ala Leu Asp Gly 75 Ala Asp Tyr Ser Gly Thr Tyr Gly Ile Thr Thr Ser Gly Asn Ser 90 Leu Arg Leu Asn Phe Val Thr Gly Ser Asn Val Gly Ser Arg Thr 105 Tyr Leu Met Ala Asp Asn Thr His Tyr Gln Ile Phe Asp Leu Leu 120 Asn Gln Glu Phe Thr Phe Thr Val Asp Val Ser Asn Leu Pro Cys 135 Gly Leu Asn Gly Ala Leu Tyr Phe Val Thr Met Asp Ala Asp Gly 150 Gly Val Ser Lys Tyr Pro Asn Asn Lys Ala Gly Ala Gln Tyr Gly 165 Val Gly Tyr Cys Asp Ser Gln Cys Pro Arg Asp Leu Lys Phe Ile 180 Ala Gly Gln Ala Asn Val Glu Gly Trp Thr Pro Ser Thr Asn Asn 195 Ser Asn Thr Gly Ile Gly Asn His Gly Ser Cys Cys Ala Glu Leu 210 Asp Ile Trp Glu Ala Asn Ser Ile Ser Glu Ala Leu Thr Pro His 225 Pro Cys Asp Thr Pro Gly Leu Thr Val Cys Thr Ala Asp Asp Cys 240 Gly Gly Thr Tyr Ser Ser Asn Arg Tyr Ala Gly Thr Cys Asp Pro 255 Asp Gly Cys Asp Phe Asn Pro Tyr Arg Leu Gly Val Thr Asp Phe 270 Tyr Gly Ser Gly Lys Thr Val Asp Thr Thr Lys Pro Phe Thr Val 285 Val Thr Gln Phe Val Thr Asp Asp Gly Thr Ser Ser Gly Ser Leu 300 Ser Glu Ile Arg Arg Tyr Tyr Val Gln Asn Gly Val Val Ile Pro 315 Gln Pro Ser Ser Lys Ile Ser Gly Ile Ser Gly Asn Val Ile Asn 330 Ser Asp Phe Cys Ala Ala Glu Leu Ser Ala Phe Gly Glu Thr Ala 345 Ser Phe Thr Asn His Gly Gly Leu Lys Asn Met Gly Ser Ala Leu 360 Glu Ala Gly Met Val Leu Val Met Ser Leu Trp Asp Asp Tyr Ser 375 Val Asn Met Leu Trp Leu Asp Ser Thr Tyr Pro Ala Asn Glu Thr 390 Gly Thr Pro Gly Ala Ala Arg Gly Ser Cys Pro Thr Thr Ser Gly 405 Asn Pro Lys Thr Val Glu Ser Gln Ser Gly Ser Ser Tyr Val Val 420 Phe Ser Asp Ile Lys Val Gly Pro Phe Asn Ser Thr Phe Ser Gly 435 Gly Thr Ser Thr Gly Gly Ser Thr Thr Thr Thr Ala Ser Gly Thr 450 Thr Ser Thr Lys Ala Ser Thr Thr Ser Thr Ser Ser Thr Ser Thr 465 Gly Thr Gly Val Ala Ala His Trp Gly Gln Cys Gly Gly Gln Gly 480
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FIG. 2

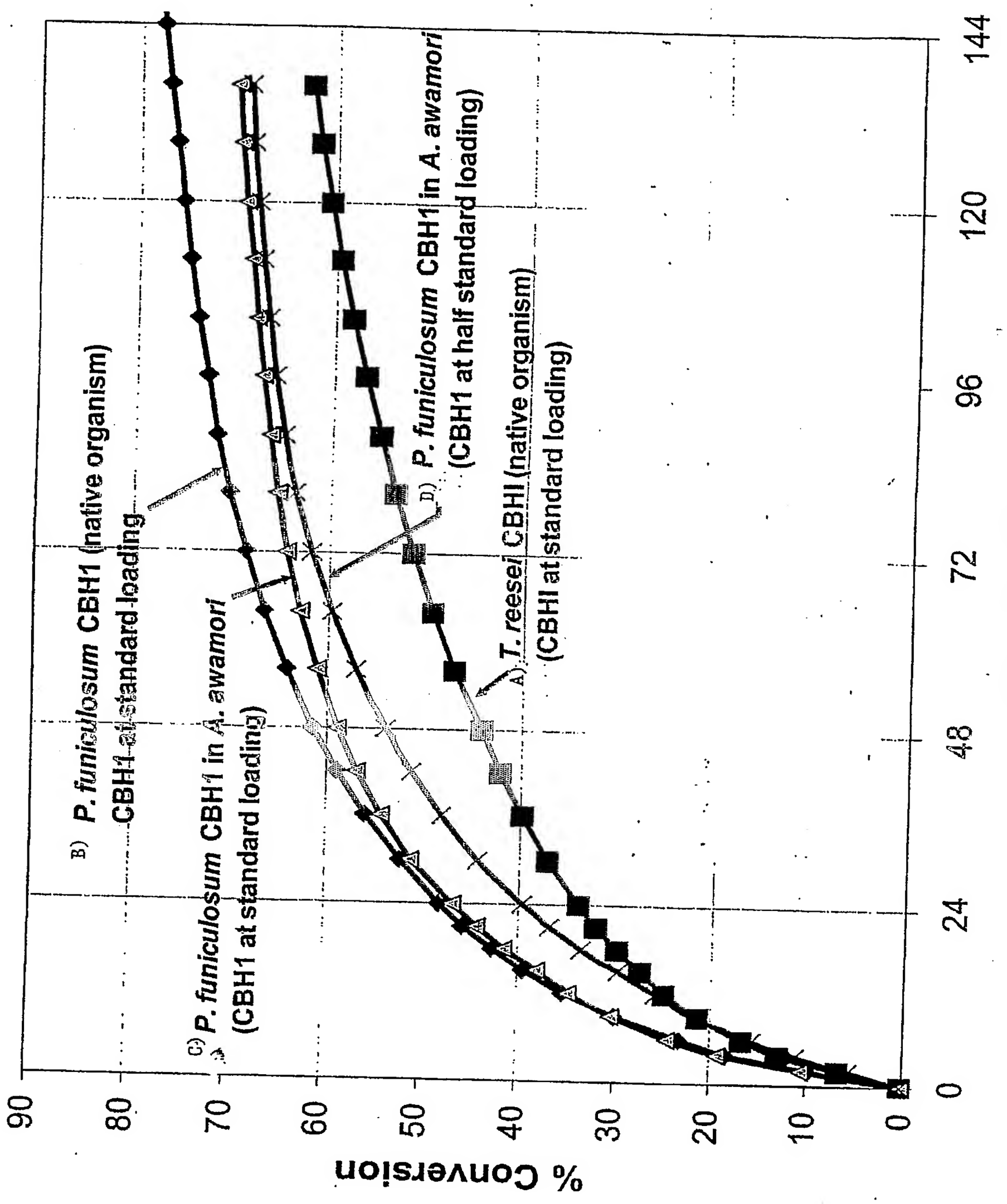

Fig. 5

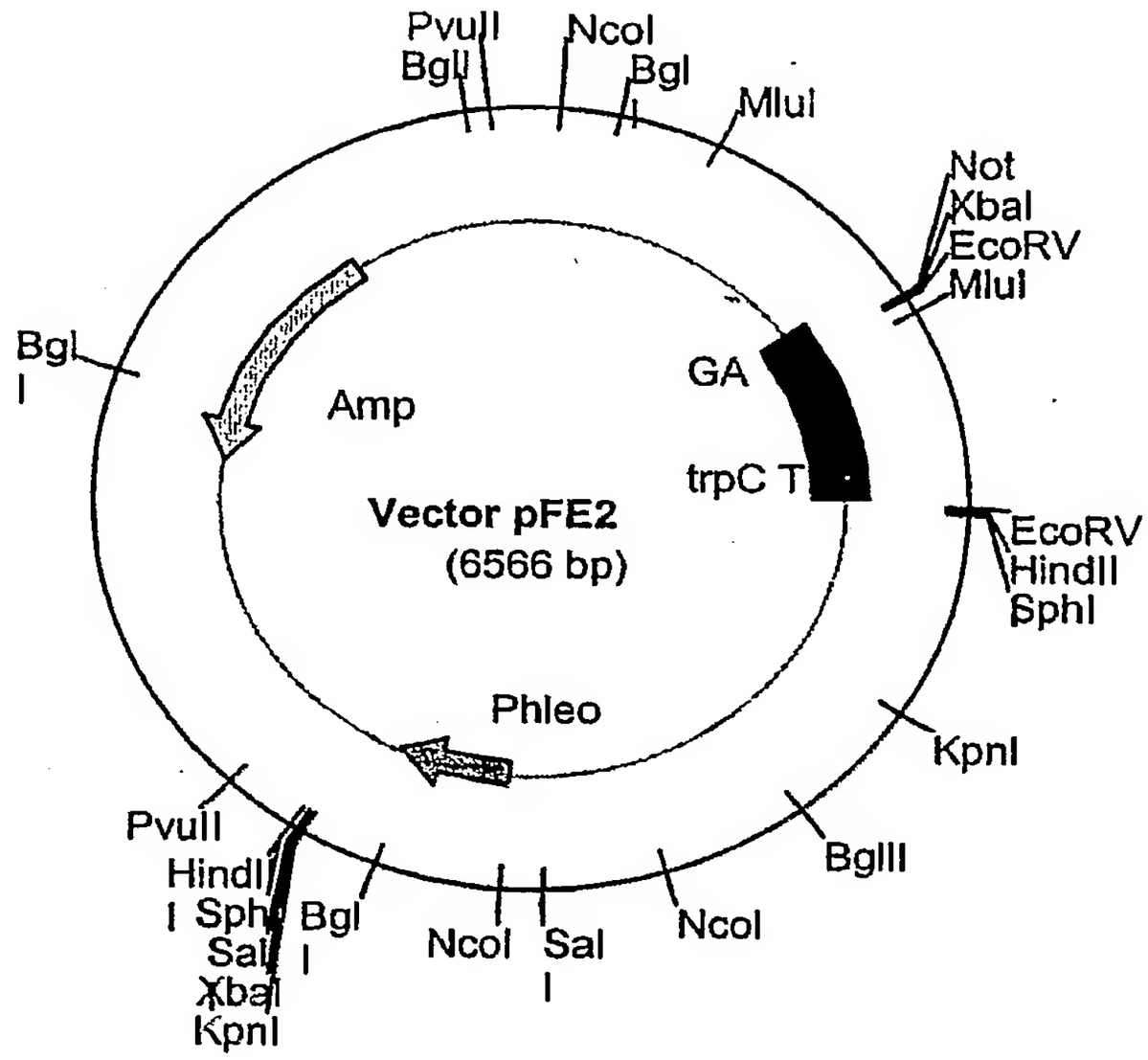

Fig. 3

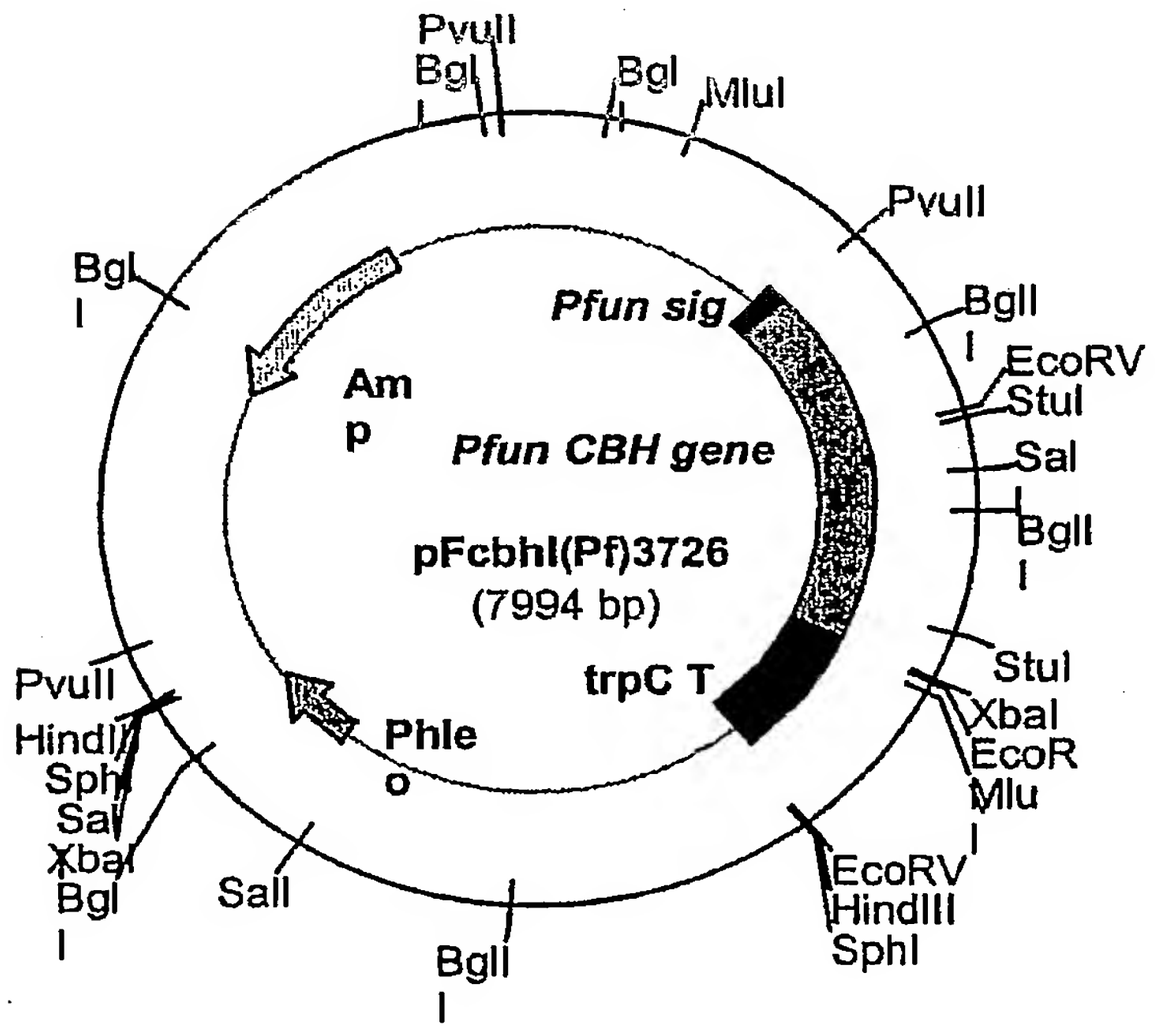

Fig. 4

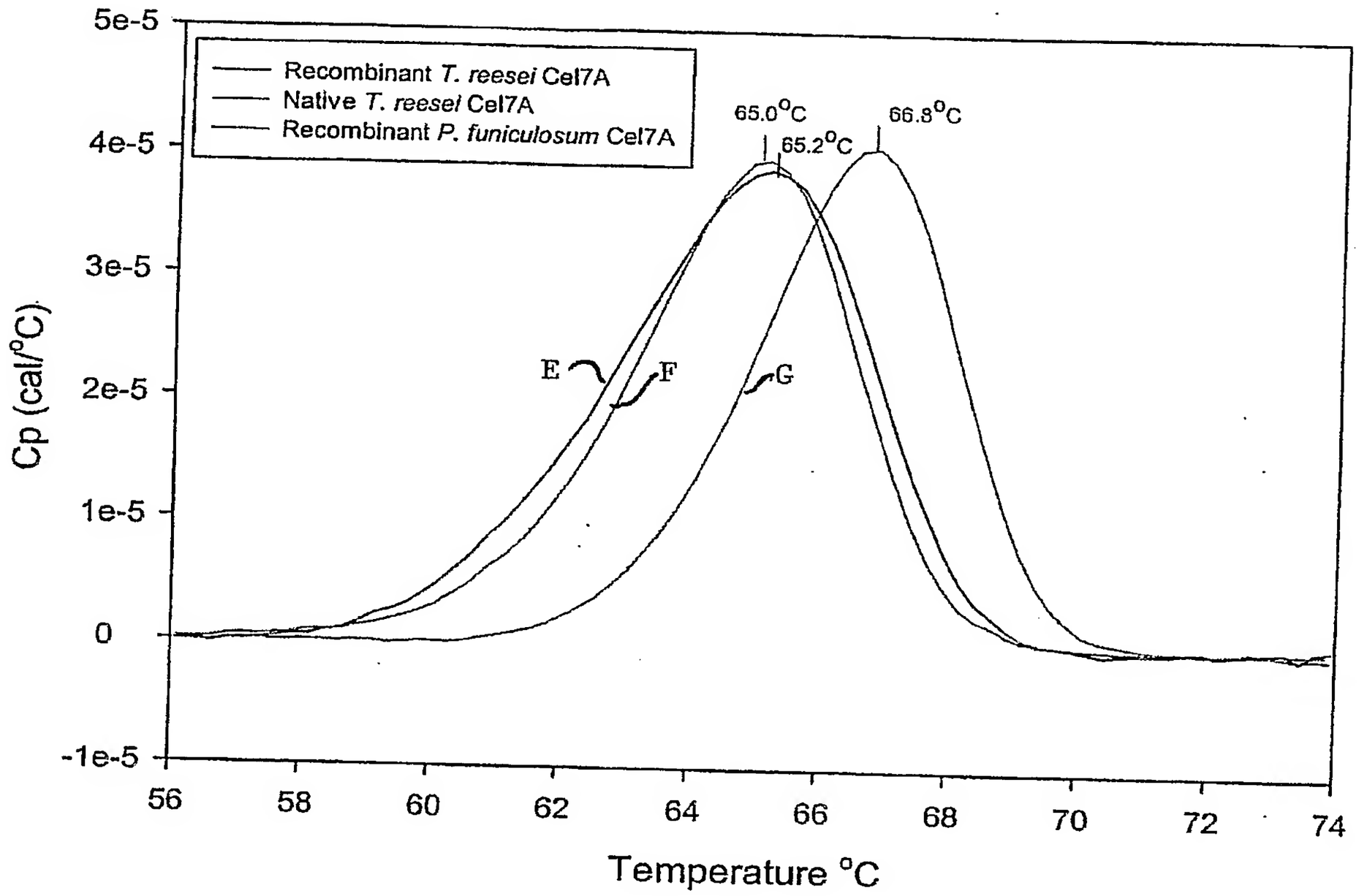

Fig. 6

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/078919 A3

(54) Title: SUPERACTIVE CELLULASE FORMULATION USING CELLOBIOHYDROLASE-1 FROM *PENICILLIUM FUNICULOSUM*

(57) Abstract: Purified cellobiohydrolase I (glycosyl hydrolase family 7 (Cel7A) enzymes from *Penicillium funiculosum* demonstrate a high level of specific performance in comparison to other Cel7 family member enzymes when formulated with purified E1cd endoglucanase from *A. cellulolyticus* and tested on pretreated corn stover. This result is true of the purified native enzyme, as well as recombinantly expressed enzyme, for example, that enzyme expressed in a non-native *Aspergillus* host. In a specific example, the specific performance of the formulation using purified recombinant Cel7A from *Penicillium funiculosum* expressed in *A. awamori* is increased by more than 200% when compared to a formulation using purified Cel7A from *Trichoderma reesei*.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/06172

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12N 9.42
US CL : 435/209
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/209

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Sequence search; CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BHAT, K.M., et al. Characterization Of The Major Endo-1,4-Beta-D-Glucanases From The Cellulase Of Penicillium pinophilum/funiculosum. Biochem. Soc. Trans. 1989, Vol. 17, No. 1, pages 103-104.	15-28
A	PARR, S.R. The Characterization Of A Commercial Cellulase Product From Penicillium funiculosum By Fast Protein Liquid Chromatography. 1985, Vol. 13, No. 2, pages 452-453.	15-28
A	WOOD, T.M., et al. The Isolation, Purification And Properties Of The Cellobiohydrolase Component Of Penicillium funiculosum Cellulase. Biochem. J. 1980, Vol. 189, No. 1, pages 51-65.	15-28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 January 2005 (13.01.2005)	10 FEB 2005
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Jamelal Shabazz for Charles L. Patterson, Jr. Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/06172

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim Nos.: 1-14 and 29-32
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Please See Continuation Sheet

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Continuation of Box I Reason 2:

Claims 1-14 and 29-32 are drawn to a formulation or a cellulase comprising SEQ ID NO:9. Applicants submitted a paper filed 11 August 2003 that stated that SEQ ID NO:1-9 are identical with the specification, namely pages 15-16 and Fig. 2. The sequence disclosure contained 10 sequences. Fig. 2 discloses that SEQ ID NO:9 is an amino acid sequence 504 amino acids long whereas the written disclosure and the CRF submitted 11 August 2003 discloses SEQ ID NO:9 as a nucleotide sequence 75 nucleotides long. Therefore no meaningful search could be done on the instant claims because SEQ ID NO:9 is not a polypeptide as in claim 1 and 29 and does not correspond to the sequence in Fig. 2.